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ORIGINAL PAPER

Study of antagonism of citric acid on aluminum-induced toxicity in mice testis cells

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Abstract To study the qualitative changes in testis tissue after aluminum chloride ($AlCl_3$) administration and to determine whether citric acid (CA) has a protective effect against testis damage induced by $AlCl_3$. In this study, 80 Kunming white mice were randomly separated into eight groups: (1) control, (2) CA (120 mg/kg), (3, 4 and 5) $AlCl_3$ (20, 40 and 60 mg/kg), (6, 7 and 8) $AlCl_3$ (20, 40 and 60 mg/kg) plus CA (120 mg/kg). After animals were killed, all testes were histopathologically examined under light microscopy; T-SOD and GSH-Px activities, H_2O_2 and MDA contents, and Bax and Bcl-2 levels were detected with the corresponding assay kits; DNA fragmentation were electrophoretically examined. Histopathological results indicated that $AlCl_3$ severely damage to mouse testis tissues, however, the protective effects on testes was observed when $AlCl_3$ combined with CA. Biochemical examination suggested that T-SOD and GSH-Px activities significantly decreased ($P<0.05$) in $AlCl_3$ groups, while remarkably improved in CA+$AlCl_3$ (especially middle and high dose groups) groups; H_2O_2 and MDA levels in higher-dose $AlCl_3$ groups were obviously higher ($P<0.05$) than in CA+ corresponding dose $AlCl_3$ groups. Cell apoptosis assays showed that Bax/Bcl-2 ratio in higher-dose $AlCl_3$ groups were higher than CA+ higher-dose $AlCl_3$ groups; and DNA fragmentation in middle-dose $AlCl_3$ groups was much more apparent in CA+ middle-dose $AlCl_3$ groups. It can be concluded that a certain concentration of $AlCl_3$ exerts a reproductive toxicity to mice, and administration of citric acid can reduce the adverse effects of $AlCl_3$ on testis.

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Aluminum (Al), one well-known metal element, is ubiquitously distributed in the environment[1]. This metal element is usually introduced to living organisms through food and drinking water. About 60% of aluminum absorbed by the body is removed from the body in the form of liquid urine, and the remainder is mainly discharged with the feces. Normally, an amount of aluminum might be contained but not accumulated in the body, thus not harmful to the body. However, with an increasing intake of metallic aluminum along with food or water, more and more researches have uniformly demonstrated that a prolonged accumulation of aluminum in the body exerts toxic effects on male reproductive system[2]. For instance, the impaired yield of spermatogonial cells after gavage administration of Al sulphate to rats has been reported in the study of Roy *et al*.[3]. Llobet *et al*. intraperitoneally injected aluminium nitrate into male mice and observed a significant reduction in the body and testicular weights, and some spermatocytes and spermatids also showed signs of necrosis[4]. Guo *et al*. found that subcutaneous administration of aluminum chloride ($AlCl_3$) resulted in a significant decrease in body weight and reproductive organ weight, the higher percentage post-implantation loss, and an severely impaired spermatogenesis within the seminiferous tubules[5]. Schrag and Dixon reported that rats received daily hypodermic injection of $AlCl_3$ for two weeks and showed the decreased sperm quality, the abnormal sperm morphology and the testicular dysfunction, which confirmed aluminum toxicity to reproductive organs[6]. As already pointed out, the type of Al compound play a critical part in determining the degree of aluminum toxicity: toxicity

of aluminum salt (i.e., Al chloride, Al sulphate) is the strongest, the next is anion-Al compounds, and the last is organic aluminum complex (i.e., Al citrate). Indeed, in the experiments by Malekshah and his coworkers, a single dose of $AlCl_3$ administered to a pregnant mouse caused fetal death and external anomalies in the fetus, whereas, Al citrate treatment exerts no adverse effects on general reproductive system[7]. However, the opinions on its toxic effects might be debatable because some studies show the toxic effects of Al citrate. For instance, in research about absorption, translocation, storage and distribution of aluminium in the body, Biyah and his colleagues demonstrated that citrate not only stimulated the intestinal absorption of Al but also induced Al accumulation within the cells[8]. The similar results have been obtained by Slanina *et al*. in 1985 as early who found an enhanced Al absorption and significantly increased serum Al concentration after treated with citric acid, and the resulting damage to the body[9]. Citric acid (CA, or called citrate) is a slightly stronger acid than typical carboxylic acids. In cellular metabolism, citric acid is the starting molecule in citric acid cycle essential for ATP production in the cell. Aerobic glycolysis has been generally accepted as a metabolic hallmark of cancers that depends on glycolysis to generate energy. Thus, glycolysis inhibition can low energy output required for neoplastic cell. And citrate (or citric acid itself) is thought of as the most effective inhibitor of glycolysis, whose ability to fight cancer by blocking glycolytic pathway has been reported in the literatures[10,11]. In vivo and in vitro studies have showed that CA or citrate has a protective role against many types of cancer[12]. According to experimental studies, the tumor-inhibiting mechanism of citric acid may be as follows: citrate can inhibit the expression of hypoxia-inducible transcription factor 1 (HIF-1), which in turn decreases the expressions of glucose transporters (e.g., glucose transporter 1), inhibitors of mitochondrial metabolism, and glycolytic enzymes including phosphofructokinase (PFK) blocking glycolysis at the start, pyruvate dehydrogenase (PDH) locating in the mitochondrial matrix, succinate dehydrogenase of citric acid cycle[13,14]. And the blocked production of glycolytic and citric acid cycle intermediates in cancer cells cause a decrease in ATP supply and furthermore triggers the intrinsic apoptosis pathway, and finally leads to cell death[12]. Stated thus, the administration of aluminum alone can adversely affect testis tissue in animals, and the mechanisms of aluminum toxicity have not yet been fully illuminated. Whether the combination of Al with citric acid can harm reproductive organ or not is still a matter of opinion. Therefore, this study was especially designed to investigate whether CA a protective effect against aluminum-induced reproductive toxicity. With these aims, the testis tissues were assayed by histological, biochemical and molecular biological methods after $AlCl_3$ and CA exposure.

Structural changes in mice testicular tissue

Light microscope (LM) examination of tissue sections of control animals stained with hematoxylin and eosin (HE) clearly illustrated the orderly arrangement of spermatogenic cells inside the seminiferous tubule of testis together with a normal presentation of interstitial cells (Figure 1A). In addition, testis structure didn't significantly change after treated with citric acid except for the loose arrangement and detachment of a few spermatogenic cells (Figure 1B). In comparison to the control group and CA group, LM examination of HE-staining testis of $AlCl_3$-treated animals revealed that seminiferous tubules was reduced in number; the basement membrane of many tubules was decayed or irregularly-shaped in appearance; some tubules appeared with wide lumen, and even entirely without spermatocytes and filled with vacuoles. Most tubules appeared with distorted and necrotic spermatocytes, the reduced number of spermatogonia, secondary spermatocytes and spermatids (Figure 1C-1E). Based on the results of HE staining, testicular tissues showed the dose-dependent damage after Al treatment. While, LM examination of HE-staining testis of peritoneally injected mice with $AlCl_3$ plus citric acid illustrated much milder histopathological changes than the corresponding $AlCl_3$ dose-treated mice, suggesting that citric acid can reduced the toxic effect of Al on testicular tissue (Figure 1F-1H).

Changes in antioxidant enzymes activities and free radical metabolites contents in mouse testis

According to the results of antioxidant enzyme activity assay, in contrast to the normal group, SOD and GSH-Px activities lowered significantly ($P<0.05$) in mouse testis after treated with CA or $AlCl_3$ individually or in combination. Furthermore, comparing with $AlCl_3$ plus CA-treated groups, $AlCl_3$ administration led to a significant ($P<0.05$) inhibition of activities of these two antioxidant enzymes in male mouse, suggesting that the animals responded more favorably to the treatment with $AlCl_3$ individually compared to the combined treatment with $AlCl_3$ and CA (Table 1). In additional, effect of CA, $AlCl_3$ either alone or in combination on H_2O_2 and MDA contents was detected. As shown in Table 1, H_2O_2 and MDA levels in the groups treated with CA, low dose of $AlCl_3$ alone or with CA were not statistically ($P>0.05$) different from that in the control group. But levels of these two metabolites were elevat-

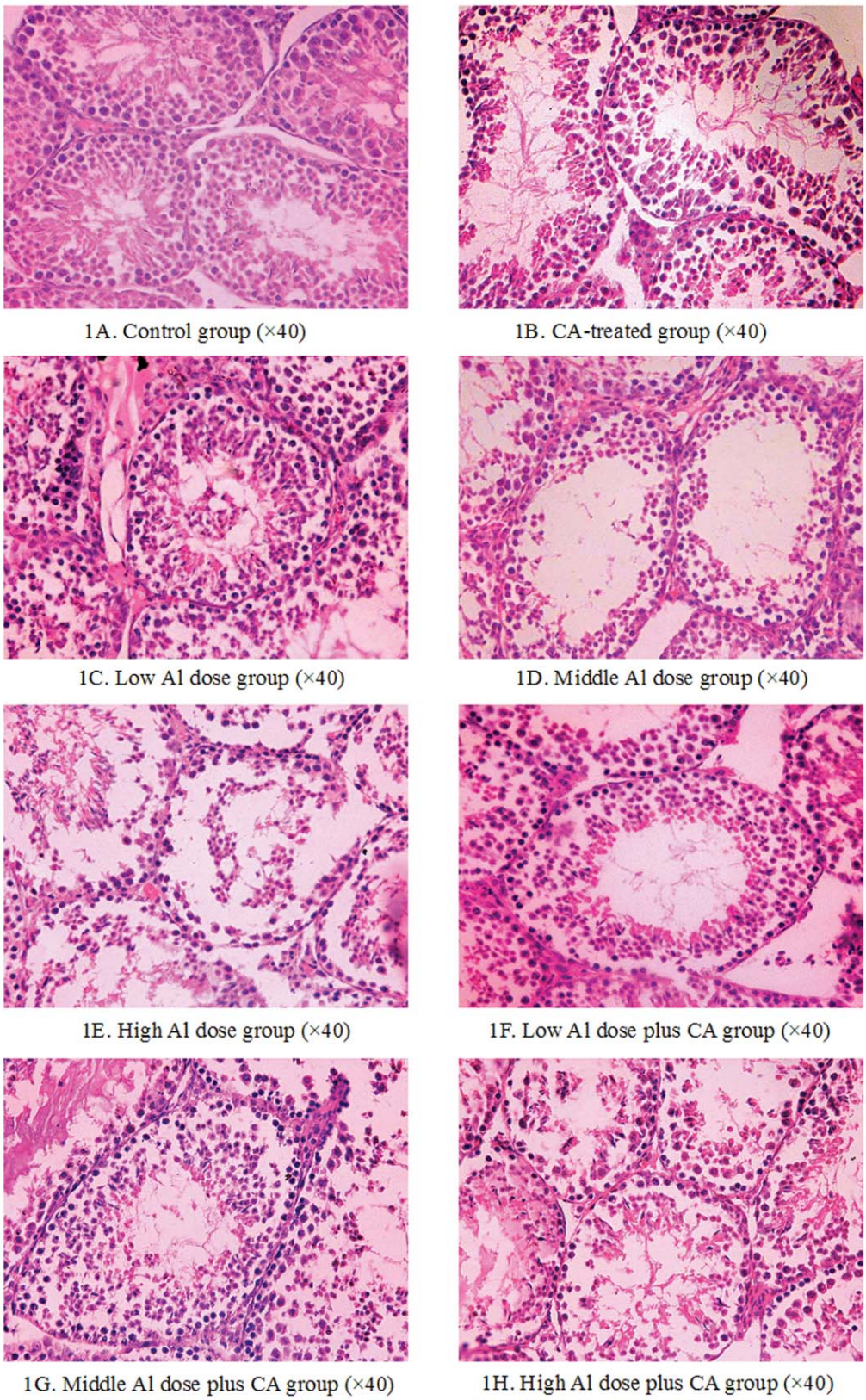

Figure 1. HE staining indicating the influence of citric acid, Al either alone or in combination on testicular tissue structure in mice.

Table 1. Effect of CA, $AlCl_3$ individually or in combination on activates of T-SOD and GSH-Px and levels of H_2O_2 and MDA in male mice.

Groups	T-SOD (U/mg)	GSH-Px (U/mg)	H_2O_2 (mmol/gprot)	MDA (nmol/mgprot)
Control	159.28 ± 3.11^{a}	27.51 ± 1.70^{a}	8.52 ± 0.49^{a}	1.50 ± 0.08^{a}
CA	149.74 ± 4.50^{b}	22.49 ± 2.46^{b}	8.45 ± 0.40^{a}	1.54 ± 0.15^{a}
Low $AlCl_3$ dose	122.92 ± 6.60^{c}	19.60 ± 2.50^{bc}	9.06 ± 0.27^{abc}	1.67 ± 0.16^{bc}
Middle $AlCl_3$ dose	108.65 ± 4.79^{d}	12.73 ± 2.47^{e}	10.35 ± 0.11^{b}	1.75 ± 0.17^{c}
High $AlCl_3$ dose	107.26 ± 6.34^{d}	13.88 ± 3.90^{de}	10.72 ± 0.27^{b}	1.71 ± 0.19^{bc}
Low $AlCl_3$ dose+ CA	119.66 ± 6.21^{c}	20.53 ± 3.09^{bc}	8.83 ± 0.13^{a}	1.57 ± 0.11^{abc}
Middle $AlCl_3$ dose+ CA	128.69 ± 9.79^{c}	19.63 ± 1.98^{bc}	9.34 ± 0.17^{cd}	1.53 ± 0.09^{abc}
High $AlCl_3$ dose+ CA	126.27 ± 6.15^{c}	17.35 ± 5.20^{cd}	9.53 ± 0.42^{cd}	1.54 ± 0.12^{ab}

Notes: Different superscript lowercase letters within the same column mean statistical significant difference between groups ($P<0.05$), same superscript lowercase letters mean insignificant difference between groups ($P>0.05$).

Table 2. Effect of CA, $AlCl_3$ individually or in combination on levels of Bax and Bcl-2 expressions in testis of mice.

Group	Bax (μg/L)	Bcl-2 (μg/L)	Bax/Bcl-2
Control	11.271 ± 0.225^{a}	105.521 ± 8.419^{a}	0.107 ± 0.004^{a}
CA	11.529 ± 0.243^{a}	94.702 ± 4.230^{b}	0.122 ± 0.002^{b}
Low $AlCl_3$ dosage	11.805 ± 0.332^{a}	93.451 ± 0.876^{b}	0.126 ± 0.003^{b}
Middle $AlCl_3$ dosage	14.329 ± 0.464^{c}	88.531 ± 3.596^{c}	0.162 ± 0.011^{d}
High $AlCl_3$ dosage	19.434 ± 0.868^{e}	84.278 ± 3.995^{c}	0.231 ± 0.001^{f}
Low $AlCl_3$ dosage plus CA	11.659 ± 0.271^{a}	95.765 ± 3.575^{b}	0.122 ± 0.004^{b}
Middle $AlCl_3$ dosage plus CA	13.209 ± 0.322^{bc}	88.427 ± 1.735^{c}	0.149 ± 0.001^{c}
High $AlCl_3$ dosage plus CA	17.757 ± 0.795^{d}	85.071 ± 0.501^{c}	0.208 ± 0.008^{e}

Notes: Different superscript lowercase letters within the same column mean statistical significant difference between groups ($P<0.05$), same superscript lowercase letters mean insignificant difference between groups ($P>0.05$).

ed significantly ($P<0.05$) in response to middle, high dose of $AlCl_3$ alone or in combination with CA when compared to the control and CA-treated group. However, H_2O_2 level in male mice treated with CA combined in higher (middle and high dose) $AlCl_3$ dose was much lower ($P<0.05$) than that in the mice treated with corresponding doses of $AlCl_3$ alone. Comparing to $AlCl_3$ treatment alone, MDA contents had a decrease in response to $AlCl_3$ plus CA. Results indicated that higher doses of $AlCl_3$ alone or with CA can cause an increase in the levels of H_2O_2 and MDA in testis tissue, but the combination of $AlCl_3$ with CA can significantly inhibit the increase in H_2O_2 level and lipid peroxidation of cellular membrane induced by $AlCl_3$ in mouse testis.

Changes in levels of Bax and Bcl-2 expressions in mouse testis

The influence of administration of $AlCl_3$ without or with CA on Bax and Bcl-2 expression in the testis of mice was evaluated using enzyme linked immunosorbent assay (ELISA) method, and the results showed that Bax level of mouse testes in response to any dosage of $AlCl_3$ were higher than that in the control and CA-treated groups, or much higher ($P<0.05$) in response to middle or high dose of $AlCl_3$. Although still higher than the control and CA-treated group, Bax level in any $AlCl_3$ dose plus CA-administered group showed a decrease in contrast to the groups treated with the corresponding dose of $AlCl_3$ alone. Furthermore, compared to high-dose $AlCl_3$ group, there was an obviously decreased ($P<0.05$) expression of Bax after treated with high dose $AlCl_3$ along with CA. As for Bcl-2, its expression level was depleted in response to any doses of $AlCl_3$ alone in contrast to the control and CA-treated groups. However, Bcl-2 level in mouse testis was slightly increased in response to the corresponding dose of $AlCl_3$ along with citric acid, albeit still lower than in the control and CA-treated groups. According to above statement, in contrast to the control and CA-treated groups, Bax/Bcl-2 ratio was increased in response to $AlCl_3$, or even significantly increased ($P<0.05$) in response to higher doses of $AlCl_3$. Whereas, this ratio was significantly lower ($P<0.05$) in response to CA ingested with higher doses of $AlCl_3$ when compared to $AlCl_3$ alone.

DNA damage in mouse testis

To confirm whether citric acid can impair the toxic effect of aluminum on mouse testicular cells, the sam-

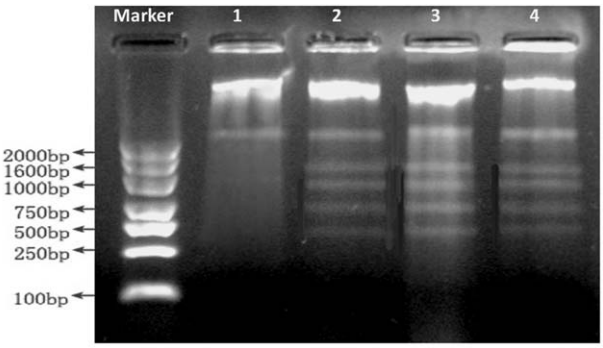

Figure 2. Testicle cells apoptosis measured by DNA Ladder Assay. Four samples representing four different treated groups were loaded in the corresponding lanes. Electrophoresis was performed on 1.2% agarose gel. 1: control; 2: CA-treated group; 3: Middle $AlCl_3$ dosage group; 4: CA plus middle $AlCl_3$ dosage group.

ples separately from the control, CA-treated, middle $AlCl_3$ dose group, and middle $AlCl_3$ dose plus CA group were picked out for DNA laddering assay. The result (Figure 2) revealed that DNA laddering, which is indicative of apoptotic events, occurred in testicle samples from three treated groups, and cell apoptosis from the control sample was not detected (Lane 1). In detail, DNA laddering was much more apparent in response to middle dose of Al (Lane 3) than that in response to middle dose of Al with CA as well as CA alone.

Discussion

Al and Al compounds are highly toxic agents that cause reproductive toxicity in animal testis tissues[15,16]. So it becomes very necessary for people to look for protective substances against reproductive toxicity of Al and Al compounds. The present work studied the influence on structures and functions of mouse testis after treatment with aluminum chloride alone or in combination with citric acid by using light microscopic examination and biochemcial analyses. In this study, light microscopic examinations showed that the injection of $AlCl_3$ alone resulted in the acute structural and histopathological changes in mouse testis, such as wrinkled seminiferous tubules shrank with the thin tubular membranes, the destruction of seminiferous epithelium, the reduced amount of various spermatogenic cells, the disordered arrangement of spermatogenic cells and even with an obvious necrosis, which suggested that aluminum exerted substantial damage on reproductive function (e.g., spermatogenesis) of male mice. Furthermore, the higher concentration of $AlCl_3$ is, the more serious injury on the structure of testicular tissue. The deformed morphology of testes recorded in our study came in consistence with the observations of Zhang *et al.*[17]. They recorded the damaged appearance in seminoma cell nuclear and the decreased number of primary spermatocytes with reduced nuclear size in rats following treatment with high dose of aluminum. These searchers attributed this negative influence of $AlCl_3$ on spermatogenesis in testis tissue to direct injury of seminiferous tubules by $AlCl_3$. It is possible to apply this explanation into the present study. Another reason might be thought to inhibit the production of androgen synthase by $AlCl_3$. Guo *et al.*'s experiments indicated that chronic Al chloride administration induced an accumulation of Al in testis, disturbed synthesis of androgen synthase, thus lowering testicular testosterone levels, finally causing the disorder of testicular spermatogenic function and the occurrence of various histopathological changes, which inferred that aluminum chloride can adversely affect the reproductive function of male animals[18]. Similarly, in the findings of Llobet *et al.*, aluminum chloride treatment has severe toxic effects on mouse's body, including presence of oedematous fluid in the tissues, irregularity of germ cell layers, pyknosis of nuclei, giant cells and degeneration with clear necrotic debris in tubules[19]. In the experiments carried out by Guo *et al.*, testicular tissues showed the dose-dependent and severe spermatogenetic impairments which were seen as necroses in spermatid cells and spermatozoa within the seminiferous tubules at 5 weeks of $AlCl_3$ treatment[5]. Generally, the structural changes in testis tissue reported in these studies are partly in agreement with the observations of our study. However, compared to treatment with $AlCl_3$ alone, the degree of testicular damage was relieved to some extent after the combined administration of $AlCl_3$ and CA, implying that treatment with this organic acid was effective in reducing toxic effect of Al on reproductive system. Coincidentally, our result was similar to findings from the study by other investigators that mice showed sperm abnormality on inorganic aluminum salt administration, but were not influenced on Al citrate treatment[7]. But just it is not enough to prove the impact of citric acid on reproductive toxicity of aluminum simply by means of histopathological examination of testis tissue, therefore, other parameters are required to test the effects of Al alone or along with CA, such as activities of antioxidant enzymes and levels of free radical metabolites which are indicative of oxidative injury of testis, and the levels of Bax and Bcl-2 expression as well as DNA laddering assessing cell apoptosis. As well-known, various reactive oxygen species (e.g., H_2O_2) are generated during xenobiotics metabolism in the body. If not terminated fast enough by enzymes such as antioxidant

enzymes including SOD, GSH-Px, there will be damage to cell membrane-locating lipids known as lipid peroxidation, which in turn destroys the structure and function of cell membranes. Meanwhile, as one of the final products of fatty acids peroxidation, Overproduction of MDA in the tissue reflects an increase in free radicals. Therefore, H_2O_2 and MDA levels are commonly known as the markers of oxidative stress and the antioxidant status. In the present study, the results showed that there was no significant change in H_2O_2 and MDA levels ($P>0.05$) after treatment with CA, low dose of $AlCl_3$ alone or with CA in contrast to the control, whereas SOD and GSH-Px activities lowered significantly ($P<0.05$) in mouse testis after suffering the same treatment. As remarked above, in our work, it appeared that a decrease in antioxidant enzymes activities after administrating low dose of $AlCl_3$ did not have obvious impact on scavenging H_2O_2 and damaging lipid structure, that is to say, low $AlCl_3$ dosage did not cause a remarkable damage to mice testis. Additionally, in this research, after treated with middle (40 mg $AlCl_3$/kg), high (60 mg $AlCl_3$/kg) dose of $AlCl_3$ alone, there was a remarkable increase in MDA and H_2O_2 levels ($P<0.05$), and an obvious decrease in T-SOD and GSH-Px activities ($P<0.05$) when compared to control and CA-treated groups, which indicated an oxidative stress following free radical generation induced by aluminum in certain dosage. The observation partly coincided with the results of Liu *et al*. that activities of serum SOD and GSH-Px correlated negatively with Al levels, in other words, SOD and GSH-Px activities in Al-loaded groups suffered the gradual reduction with the increase of Al intake[20]. Our results also suggested that, compared to treatment with a higher dose of $AlCl_3$ alone, a combined administration of the corresponding $AlCl_3$-dose and CA reduced some biochemical variables (particularly H_2O_2 ($P<0.05$)) indicative of oxidative stress. This is perhaps due to the tendency of CA binding aluminum to form Al citrate complex that can easily be eliminated through urine. Also, citric acid may act as an adjuvant that might help in removing aluminum from the deposit site. It is possible that this removal may cause the moderately decreased oxidative stress in testis tissue. This hypothesis is supported by the observations that citrate significantly enhanced plasma contents and excretion of aluminum in rabbits[21]. As stated previously, if oxidants such as hydrogen peroxide couldn't be cleared due to the low activities of antioxidant enzymes such as T-SOD or GSH-Px, subsequently, lipid superoxidation and cell apoptosis would be triggered. So, we further examined the alteration of Bax and Bcl-2 expressions, and the degree of DNA damage, indicative of cell apoptosis, thus finding out the underlying mechanism of the toxic effect of Al on testis tissue. As popularly accepted, whether a cell becomes committed to apoptosis partly depends on the balance between pro-apoptotic proteins (like Bax) and anti-apoptotic proteins (like Bcl-2). Bax overexpression can accelerate cell death, an effect that is associated with formation of Bax/Bax homodimers. Conversely, Bcl-2 overexpression can repress the pro-death function of Bax, which is associated with formation of Bcl-2/Bax heterodimers. Thus, Bax/Bcl-2 ratio becomes a crucial determinant of the fate of a cell toward survival or apoptosis. It is generally accepted that the higher Bax /Bcl-2 ratio, the lower cell survival rate. For instance, in Wang *et al*.'s study on the effect of $AlCl_3$ on apoptosis of rat hippocampus cells and the relationship between apoptosis and expression of Bcl-2 as well as Bax, and result showed that, with the increasing dosage of Al treatment, apoptotic index of the neurons became higher, accompanied by the significantly increased Bax expression, significantly decreased Bcl-2 expression and the higher Bax/Bcl-2 ratio. Accordingly, they concluded that aluminum might induce hippocampus cell apoptosis in rats through up-regulating Bax and down-regulating Bcl-2[22]. Our study also observed the similar result that there was a gradually increasing expression of Bax and a gradually decreasing expression of Bcl-2 in testis tissue of mice with the increasing dosages of $AlCl_3$, also indicating a dose-effect relationship between them. However, we found that inhibition of Bcl-2 expression was improved and upregulation of Bax expression was attenuated after treatment of Al with citric acid when compared to Al treatment alone, and this change was especially evident for high dose group. At the same time, the combined administration of higher dose $AlCl_3$ and CA resulted in a marked reduction in Bax/Bcl-2 ratio compared to the corresponding dose of $AlCl_3$ alone. In this connection, it could be considered that citric acid can form complexes with Al and thus reduce the inductive effect of this metal on cell apoptotic death. Since the exposure of cells to cytotoxic agents is often associated with morphologic changes of apoptosis and, in some cases, DNA fragmentation, in this study, DNA laddering assay was performed on the testes samples. Also, based on above biochemical test result that low dose of $AlCl_3$ did not cause a remarkable damage to mice testis, and higher dose of $AlCl_3$ exerted obviously damage to testis tissue; however, there is no significant difference in free radical metabolite levels and antioxidant enzyme activities between middle dose group and high dose group. Therefore, in this study we choose the testes samples from control, CA, middle $AlCl_3$ dose, and middle $AlCl_3$ dose plus CA groups to have a DNA ladder test. We observed the formation of DNA laddering in testicle

samples from other three treated groups except the control, suggesting that cell apoptosis occurred in response to citric acid, Al either individually or in combination. Whereas, DNA laddering was much more apparent in response to middle Al dose than that in response to middle dose of Al with CA as well as CA alone, reconfirming the conclusion drawn from our data above. In summary, the present study suggests that the higher doses of $AlCl_3$ can produce toxic effects on mouse testis possibly through directly damaging the structure of testicular tissue, inhibiting the activities of antioxidant enzymes, and inducing cell apoptosis; whereas, citric acid can decrease to some extent the toxicity of Al on testis. Thus, it can be concluded that CA could be a potential antidote for aluminum accumulation.

Materials & Methods

Chemicals and reagents

Citric acid were purchased from Tianjin Fu Chen Chemical Reagents Factory (Tianjin, China). Aluminum chloride was obtained from Tianjin Damao Chemical Reagent Factory (Tianjin, China). T-superoxide dismutase Assay Kit, Glutathione Peroxidase Assay Kit, Hydrogen Peroxide Assay Kit and Maleic Dialdehyde Assay Kit were bought from NanJing JianCheng Bioengineering Institute (NanJing, China), and Bcl-2, BAX Elisa Kits were from Beijing Dingguo Changsheng Biotech Co. Ltd (Beijing, China).

Chemical administration to animals

In this study, 80 adult male Balb/C albino mice, weighing 20-24 g, obtained from the Animal Center of Medical School of Henan University of Science and Technology (Admission number of animal use: SYXK (Henan) 2008-0105). Animals were housed in stainless steel cages in an air-conditioned room, and allowed free access to feed and water during the whole period of the experiment that lasted on the 28th day. All experiments were strictly done in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China.

These animals were randomized into eight groups, each with 10 mice, including control (0.9% saline), 120 mg CA/kg, 20 mg $AlCl_3$/kg, 40 mg $AlCl_3$/kg, 60 mg $AlCl_3$ /kg, 20 mg $AlCl_3$/kg+120 mg CA/kg, 40 mg $AlCl_3$/kg+120 mg CA/kg, and 60 mg $AlCl_3$/kg+120 mg CA/kg body weight, respectively[15]. Animals in each group received the peritoneal injection of corresponding reagents once a week for 3 weeks. The same volume of 0.9% saline solution was given as a solvent to the control group during 3 weeks. At the 7th day since 3rd injection, all the animals were killed by neck dislocation. All testes were dissected out immediately, cleaned and processed immediately for following various estimations.

Histological examination of testis tissues

A certain amount of testis samples were taken and cut into 3 mm × 4 mm blocks. After 10% neutral formalin fixation for 48 h, samples were embedded in paraffin and 3 μm sections were prepared for HE staining: dewaxing with xylene, rehydration with graded alcohol (100%-50%), hematoxylin for 4 min, running tap water for 1 min, eosin for 10 min, 70% ethanol for 1 min, 95% ethanol for 1 min, 100% ethanol for 1 min, two rinses in 100% xylene for 2 min each. Finally, morphological changes of testis tissues of eight groups were observed under light microscope (LM).

Determination of activities of T-SOD, GSH-Px and contents of H_2O_2, MDA

Firstly, 10% testis homogenate of mouse were prepared. Then, activities of T-SOD, GSH-Px and contents of H_2O_2, MDA in testis tissue were tested according to guideline from the corresponding assay kits[23].

Elisa assay for Bcl-2 and Bax expression levels

Testis tissues were prepared into a homogenate, diluted 10 times with distilled water and centrifuged at 3000 r/min for 15 min. And the supernatant was collected for use. Coated wells were washed four times, mixed with 50 μL of enzyme conjugate and incubated at 37°C for 30 min. The wells were washed five times, sequentially mixed with 50 μL of chromogenic agent A and 50 μL of agent B, gently shaken for 30 sec and colorized in the dark at 37°C for 15 min. The reaction was terminated with stop buffer. Absorbance of each well at 450 nm was measured with a microplate reader (Thermo Fisher Scientific Oy, Finland). The linear regression equation of standard curve was calculated according to standard sample concentration and the corresponding absorbance. Sample concentration was calculated using regression equation according to sample absorbance. Final concentration was determined by multiplying the sample concentration by the dilution multiple[5].

DNA ladder assay

The samples in the control, CA group, middle $AlCl_3$ dose group, and middle $AlCl_3$ dose plus CA group were randomly selected. A certain weight of testis tissue was taken and cut into small pieces. DNA isolation was

performed according to the protocol introduced in DNA Ladder Detection Kit. The extracted DNA was electrophoresed in 1.2% agarose gel for assaying DNA fragmentation.

Statistical analysis

All results were expressed as mean ± SD and analyzed by SPSS version 18.0 (SPSS, Chicago, IL, USA). All data were analyzed using a one-way analysis of variance (one-way ANOVA) with least significant difference (LSD) multiple comparison tests to identify significant differences between different groups. A value of $P < 0.05$ was considered statistically significant.

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Conflict of Interest The authors declare no conflict of interest.

References

1. Colak, S. *et al*. The neuroprotective role of boric acid on aluminum chloride-induced neurotoxicity. *Toxicol Ind Health* **27**:700-710 (2011).
2. Guo, C. H. *et al*. The influence of aluminum exposure on male reproduction and offspring in mice. *Environ Toxicol Pharmacol* **20**:135-141 (2005).
3. Roy, A. K. *et al*. Similar effects in vivo of two aluminum salts on the liver, kidney, bone, and brain of Rattus norvegicus. *Bull Environ Contam Toxicol* **47**:288-295 (1991).
4. Llobet, J. M. *et al*. Reproductive toxicology of aluminum in male mice. *Fundam Appl Toxicol* **25**:45-51 (1995).
5. Guo, C. H. *et al*. Aluminum-induced suppression of testosterone through nitric oxide production in male mice. *Environ Toxicol Pharmacol* **19**:33-40 (2005).
6. Schrag, S. D. & Dixon, R. L. Occupational exposures associated with male reproductive dysfunction. *Annu Rev Pharmacol Toxicol* **25**:567-592 (1985).
7. Malekshah, A. K. *et al*. Developmental toxicity of aluminum from high doses of $AlCl_3$ in mice. *J Appl Res* **5**:575-579 (2005).
8. Biyah, K. *et al*. SR 140333 prevents potentiation by citric acid of plasma exudation induced by histamine in airways. *Eur J Pharmacol* **308**:325-328 (1996).
9. Slanina, P. *et al*. Influence of dietary factors on aluminium absorption and retention in the brain and bone of rats. *Acta Pharmacol Toxicol* **56**:331-336 (1985).
10. Wang, Q. Q. *et al*. Anticancer based on glycolysis pathway in tumor cells. *Int J Oncol* **V38**:914-916 (2011).
11. Halabe Bucay, A. Hypothesis proved...citric acid (citrate) does improve cancer: a case of a patient suffering from medullary thyroid cancer. *Med Hypotheses* **73**:271 (2009).
12. Zhou, Z. *et al*. Inhibitory effect and mechanism of citrate on cylgolysis in human gastric cancer cell line SGC7901. *Chin J Gastroenterol* **18**:271 (2013).
13. DeBerardinis, R. J. *et al*. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci USA* **104**:19345-19350 (2007).
14. Halabe Bucay, A. The biological significance of cancer: mitochondria as a cause of cancer and the inhibition of glycolysis with citrate as a cancer treatment. *Med Hypotheses* **69**:826-828 (2007).
15. Shen, W. G. *et al*. Effect of aluminum on meiotic maturation of mouse oocyte in vitro. *J Hyg Res* **28**:267-268 (1999).
16. Cui, H. H. *et al*. Toxicity of aluminum trichloride in male mice. *J Environ Health* **26**:68-70 (2009).
17. Zhang, M. X. *et al*. The effect of aluminum of seminiferous tubules in rat testes. *Chin J Ind Hyg Occup Dis* **9**:38-40 (1991).
18. Guo, C. H. *et al*. The influence of aluminum exposure on male reproduction and offspring in mice. *Environ Toxicol Pharmacol* **20**:135-141 (2005).
19. Abdel-Moneim, A. M. Histological study of the effect of aluminum in testes of albino mice. *MRVSA* **2**:52-58 (2013).
20. Liu, P. *et al*. Effects of aluminum on trace element contents and anti-oxidation enzyme system in serum and brain of rabbits. *J Shandong Univ* (*Health Sciences*) **40**:324-325, 328 (2002).
21. Fulton, B. & Jeffery, E. H. Absorption and retention of aluminum from drinking water. 1. Effect of citric and ascorbic acids on aluminum tissue levels in rabbits. *Fundam Appl Toxicol* **14**:788-796 (1990).
22. Wang, L. P. *et al*. The effect of aluminum on the apoptosis of hippocampus cell and the expression of the Bcl-2 and Bax in rats. *J Environ Occup Med* **22**:225-228 (2005).
23. Chen, X. *et al*. Study on injury effect of food additive citric acid on liver tissue in mice. *Cytotechnology* **66**: 2752-2782 (2014).